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TITLE: The Role of Focal Adhesion Kinase and CAS in Integrin-medicated Signaling on Distinct Forms of Laminin-5

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This report details final work on this project that was performed under a revised statement of work at BioForce Nanosciences, Inc. Ames IOWA. The goals of this project were to test the feasibility of creating ultraminiaturized protein arrays using the BioForce Nanoarrayer™ instrumentation for the long term goal of providing a platform for screening biomarkers during progression in human breast cancer cell lines. The results of this study prove feasibility of this method and provide the groundwork for an assay format that will be useful for conducting biomarker profiling studies from exceedingly small tissue biopsy samples, such as those obtained from laser capture microdissection.

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Introduction and Personal Statement:

This award was transferred to a new laboratory, BioForce Nanosciences, in Ames Iowa following the closure of the original laboratory at the University of Nevada, Las Vegas. The new title was: Protein NanoArrays for Profiling Malignant Progression in Breast Cancer Cell Lines. The following report is the final summary of work performed after the transfer to BioForce Nanosciences. The final report from the work performed at UNLV was previously submitted in May 2003 and received approval. A copy of this report is attached at the end of this new report in the appendix. The transfer of the award and the commencement of the new work plan began in November 2002 and terminated June 30, 2003.

Revised Statement of Work:

Introduction: The goal of this phase of research was to test the feasibility of utilizing the BioForce NanoArrayer for the construction of ultraminiaturized "reverse-phase" protein nanoarrays for profiling malignant progression in breast cancer cell lines. Briefly, the nanoarrayer is a novel surface patterning instrument, built from the ground-up at BioForce Nanosciences, which can be used for the construction of arrays with domain sizes in the micron to submicron spatial scale. The NanoArrayer utilizes controlled mechanical deposition of solutions onto a surface with high spatial resolution, surface sensing capability and precise environmental control of the ambient temperature and humidity. A deposition tool is loaded by immersion of its distal end in a spot of sample solution, followed by time-controlled contact of the tool with the surface to be patterned. Standard, state of the art microarray spot sizes range anywhere from 30 μm to 150 μm. These nano-scale arrays allow a thousand or more molecular tests to be carried out in the same surface space occupied by a single state-of-the-art microarray spot. An obvious advantage of this design is the dramatic savings in reagent cost that accompanies ultra-miniaturization. More importantly, it provides a realistic platform for performance of array-based analyses in applications involving extremely small quantities of sample material where uses of current microarray formats are not feasible. Protein profiling of laser capture microdissected samples from human tissue biopsies is an example of such an application The goal of this research project was to adapt our NanoArray technology for use as a platform for measuring protein profiles in human breast cancer cell lines. The ultimate use of this technology will be for identification and monitoring protein biomarker profiles during pre-malignant cancer progression and for therapeutic monitoring in human biopsy samples.

Task 1: Determine precision and linearity of NanoArray platform using purified antigen.

1) A suitable protein, such as recombinant human estrogen receptor (#RDI-ERALPHA-AG Research Diagnostics Inc, NJ) will be arrayed in dilution curves on NanoArray chips and will be detected with an anti-estrogen receptor antibody (Research Diagnostics Inc., NJ), followed by a fluorescently labeled secondary antibody.

From these control experiments we will determine: Intra- and Inter-spot reproducibility, limits of protein detection, and linearity of detection.

Task 1 Progress Report 11/01/02 – 6/30/03:

This task has not been completed to date. I started with task 2, to work out conditions for arraying cell lysates.

Task 2: Determine precision and linearity of NanoArray using protein lysates from breast cancer cell lines.

1) Defined cell lysates from standard breast epithelial cell lines representing various stages of clinical breast cancer progression (normal: MCF10A; MCF-7, MDA-MB-231 and MDA-MB-425) will be prepared for use in this phase of assay optimization.
2) Salt, protein and detergent concentrations may be altered to achieve optimal protein deposition.
3) Protein profiles of previously validated markers proteins will be generated during this phase of research. These markers will include Estrogen Receptor, Progesterone Receptor and HER2/*neu*.

Task 2 Progress Report 11/01/02 – 6/30/03:

For speed and simplicity in this phase of assay development I used the established human cell line A431 that overexpresses the Epidermal Growth Factor Receptor for assay optimization. This cell line, along with antibodies to the EGFR where already available at BioForce.

This task was divided into two interrelated subtasks that constituted the bulk of the work completed on this project:

1) Determine optimal substrate for lysate binding
2) Determine optimal lysis buffer conditions

A. Surface optimization:

The following surfaces were tested for protein immobilization:

1) Bare gold
2) Gold containing an amine-reactive self assembled monolayer. (DSU; Dithiobis succinimidyl undecanoate)
3) Hydrogel 3-D matrix (Packard)
4) Epoxy and Aldehyde surfaces (Telechem International)
5) FastSlides (Schleicher and Schuell), glass-backed nitrocellulose slides
6) Thin layer nitrocellulose (made in house)
7) Versalinx chemistries (Prolinx)
8) Power matrix slides (Full Moon Biosystems)

DSU surfaces were determined to be the best of those tested. These were assembled on 4 X 4 mm polished silicon chips (Montco Silicon Technologies, Inc.; PA) that were coated with 5 nm Cr and 10 nm Au using an ion beam sputterer. To facilitate array alignment, alphanumeric indexed electron microscopy grids were placed on each chip prior to sputtering (Electron Microscopy Sciences; PA). Sputtered chips were immediately immersed in a 0.5 mM solution of DSU (dithiobis-succinimidyl undecanoate, DSU; Dojindo Molecular Technologies, Inc., MD) dissolved in 1,4 dioxane, and incubated for 3 hours at room temperature. Chips were washed and briefly sonicated in 1,4 dioxane, blown dry, and stored at room temperature under dry N_2 gas.

B. Sample Preparation.

A variety of cell lysis conditions were tested. Factors considered were compatibility with nanoarray surface, compatibility with nanoarray deposition process and optimal protein extraction. I arrived at three conditions that are compatibile with the amine-reactive DSU surfaces, that work with the deposition tools and that provide optimal protein extraction.

1) Phosphate-buffered RIPA. This is the standard protein extraction buffer RIPA, altered to contain a phosphate buffer (DSU surfaces are not compatible with Tris); and lower concentration of SDS, DOC and NP-40 detergents.

2) T-Per Tissue extraction buffer from Pierce.

3) N-octyl glucoside lysis buffer (50 mM NaPhosphate pH 7.4, 1% NOG, 100 mM NaCl,).

The procedure for testing buffer conditions was as follows:

Surface compatibility:

Simple, macroscale assays were performed to test compatibility of lysis buffers with DSU surfaces. Briefly, DSU monolayers were constructed on silicon chips as described above, except the chips were sputtered through an electron microscope grid containing a single 0.6 mm central target. Rabbit IgG was diluted into each of the buffers at a final concentration of 0.1 mg/ml and 1 µl of each solution was pipeted on to the 0.6 mM gold pad on individual chips. The positive controls for these experiments were rabbit IgG diluted in standard PBS and 10:10 buffer (10mM TrisHCl pH 7.5, 10 mM NaCl). These chips were processed as follows: Protein binding to DSU was facilitated by incubation of the surface in a humid chamber for 1 hour at room temperature. The chips were then washed in PBS containing 0.2% Tween-20 (PBS-T), blocked in 1X ViriBlock™. Bound rabbit antibodies were detected by incubation with Alexa-594 goat anti-rabbit secondary antibodies (Molecular Probes, OR). Following 3 successive washes in PBS-T, chips were analyzed on a Nikon TE-2000 inverted microscope equipped with a Texas Red filter (#41004, Chroma Technology). Images were captured using an Orca mega-pixel cooled CCD camera (Hamamatsu) and MetaVue image processing software (Metamorph). The results from one of these studies conducted on bare gold surfaces are shown in Figure 1. After analysis using macroscale assays, each buffer condition was tested to determine optimal protein deposition from the NanoArrayer deposition tool onto a given surface (Examples are shown in Figure 2). In addition, each lysis buffer condition was tested to analyze protein extraction using standard Western blotting techniques and probing with markers for transmembrane proteins, peripheral membrane proteins and cytosolic

proteins (data not shown). Ultimately, I arrived at DSU surfaces and P-RIPA buffer as a good combination for construction of nanoarrays.

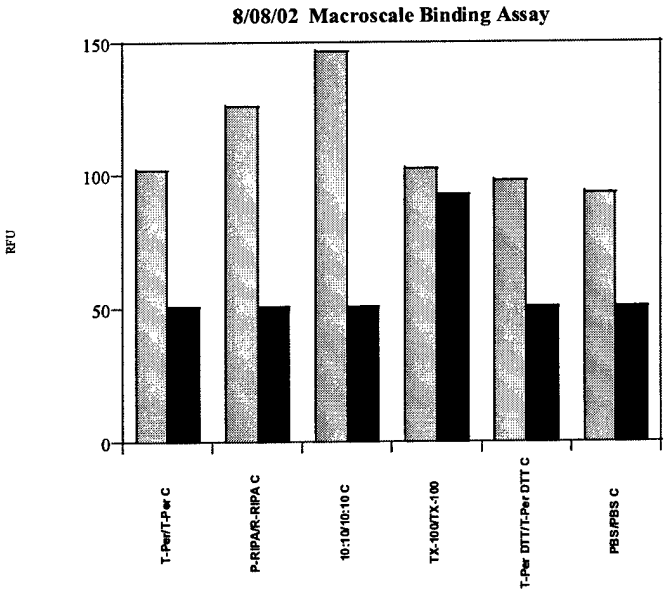


Figure 1. Macroscale assay showing protein binding on bare gold surface. In this particular experiment rabbit IgG was diluted into various buffers and then tested for binding on bare gold surfaces. Buffers for this experiment were T-Per, Phosphate-buffered RIPA (P-RIPA), 10:10 buffer, 1% TX-100 in PBS, T-Per containing 10 mM DTT, and PBS. 1 µl of a 0.1mg/ml solution of rabbit IgG was pipetted onto the central target of a gold coated 16mm2 silicon chip and incubated in a humid chamber for 1 hour at room temperature. Chips were washed in PBS-T, blocked in 1X ViriBlock buffer, and protein binding was detected by incubation with Alexa 594 goat anti-rabbit. Black bars are negative control data obtained from identical chips incubated with buffer minus rabbit antibody and processed in the same fashion.

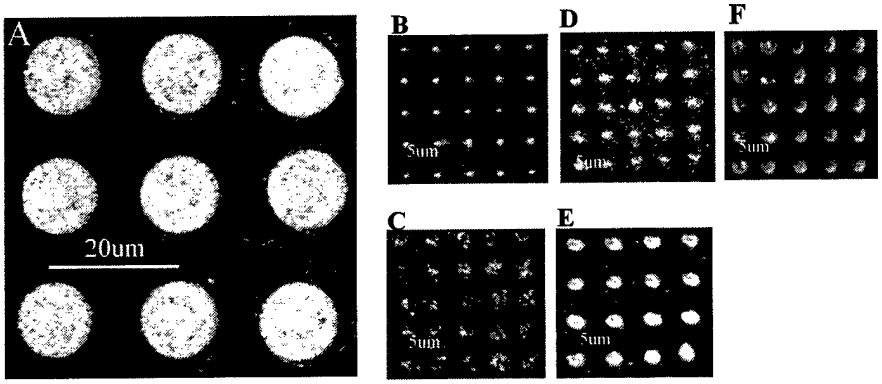


Figure 2. Examples of Nanoarrays writing on various surfaces. Panel A shows a 3 X 3 array with a 20um pitch (spot to spot spacing) of Rabbit IgG in T-per buffer written on Hydrogel 3-D matrix (Packard). Panel B was Rabbit IgG in T-Per buffer written on a bare gold surface; Panel C is Rabbit IgG in T-Per buffer containing 10% glycerol written on bare gold; Panel D shows Rabbit IgG in T-Per written on DSU; Panel E shows Rabbit IgG in T-Per containing 10% glycerol written on DSU; Panel F shows rabbit IgG in P-RIPA written on DSU. All chips were processed as described above and protein binding on the surface was detected using Alexa 594 goat anti-rabbit secondary antibodies.

In the next phase of assay optimization nanoarraying experiments were performed using total cell lysates obtained from A431 or Hela cell lines which were "spiked" with rabbit

IgG (See Figure 3 for examples), using T-Per, P-RIPA and N-octyl glucoside lysis buffers on DSU surfaces. P-RIPA lysates mixed with glycerol performed optimally for this analysis. Figure 3 shows the results of an arraying experiment where a lysate from A431 cells extracted with P-RIPA buffer was arrayed on a DSU surface and interrogated with an antibody against the human EGFR. This data demonstrates the feasibility of utilizing this "reverse-phase" nanoarray for profiling biomarkers from human cell line lysates.

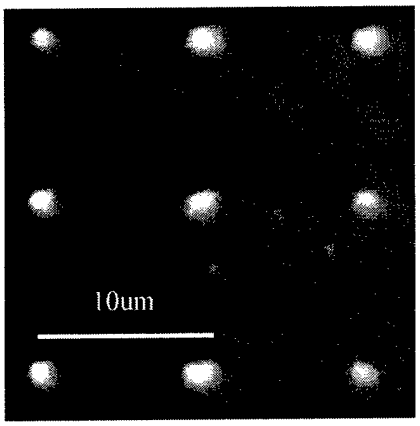


Figure 3. Reverse-Phase Nanoarray. Lysates from the human cell line A431 lysed in P-RIPA containing protease inhibitors, were deposited using the Nanoarrayer onto a DSU surface prepared on a gold-sputtered 4X4 mm silicon chip. After humidification and incubation to facilitate protein binding onto the DSU surface, the chip was probed with a goat antibody against human EGFR. Binding events were detected by incubation with Alexa 594 donkey anti-goat secondary antibodies. The image was captured as described in progress report for specific aim 2. The spot size in this experiment was approximately 2 microns with a pitch of 10 microns.

Task 3: Study expression patterns of pro-survival markers in breast cancer progression cell line model.

1) Cell lysates will be prepared, arrayed and processed according to conditions established upon successful completion of Specific Aims 1 and 2.

3) Identically arrayed protein chips will be probed with monoclonal antibodies for pro-survival markers. Specifically, we will assess expression levels and phosphorylation states of Protein

Task 3 Progress Report November 1 2002 – June 30 2003:

Due to time and budget constraints, this phase of the study was not completed.

Key Accomplishments November 1 2002 – June 30 2003:

- Demonstration of feasibility of utilizing nanoarrayer platform for construction of reverse-phase nanoarrays using human cancer cell line A431.
- Established collaboration with FDA-NCI clinical proteomics program for continued development of this procedure. They are excited and willing to provide laser capture microdissected tumor tissue samples for further evaluation when I reach the point where assay development ready for real samples.
- Presentation of data at 3 national and international conferences, listed below.
- Publication of manuscript cited below.

Reportable Outcomes November 1, 2002 – June 30, 2003.

Publications, Abstracts and Invited Talks: (all with acknowledgement of support from this Army award):

Publications:
Lynch, M., Mosher, C., Huff, J., Nettikadan, S., Johnson, J., and Henderson, E. Functional protein nanoarrays for biomarker profiling. Accepted by *Proteomics*, 2003.

Abstracts:
Huff, J.L., Lynch, M.L., Mosher, C., Nettakadan, S., Johnson, J., and Henderson, E. Protein NanoArray for Profiling Human Breast Cancer Progression Era of Hope 2002 Orlando FL.

Huff, J.L., Lynch, M.L., Mosher, C., Nettikadan, S., Johnson, J., and Henderson, E. Practical Approaches to Nanoscale Biodiagnostics. Granada Spain July 2003.

Kristmundsdottir, A., Huff, J.L., Lynch, M.L., Mosher, C., Nettikadan, S., Johnson, J Cao, C., Ryckman, K., Radke, C., Vengasandra, S. and Henderson, E. NanoArray Technology for Biomolecular Analysis. BioArrays Europe, Cambridge, UK September 2003.

Invited Talks:
NanoArray Technology in Cancer Diagostics. UCSF Cancer Center February 2003.

BioArrays Europe Practical Approaches to Nanoscale Biodiagnostics September 2003 Cambridge, UK.

Nanoscale Assays for Cancer Diagnostics. Cancer Drug Research & Development conference San Diego, CA November 2003.

Conclusions: The goal of this revised statement of work was to construct a nano-scale protein array platform and use it as a basic research tool to study alterations in cellular signaling pathways that accompany breast cancer disease progression. In the long term we anticipate that this assay will provide a much needed platform for breast cancer proteomics. I believe that significant progress towards achieving this goal was made during the course of this project. I have formed a collaboration with researchers at the FDA-NCI clinical proteomics program who are willing to provide human tissue biopsy samples from breast cancer patients, dissected using laser capture microdissection, for analysis using this platform. This project will continue if and when I am able to secure outside funding for the research.

Introduction and Personal Statement:

This award has now been transferred to a new laboratory, BioForce Nanosciences in Ames Iowa. The new title is: Protein NanoArrays for Profiling Malignant Progression in Breast Cancer Cell Lines. The following report is the final summary of work performed under the previous title as described below. This work was carried out over the 18 month time period from June 30, 2000 to December 15, 2001. This work was abruptly interrupted when my old advisor moved to a new location. I submitted my yearly report in July of 2001, and my lab was being shut down by August of 2001. I remained until December of 2001 and tried to complete as much of the work as possible, before moving myself. Unfortunately, given the circumstances of the move and the fact that this project was fraught with multiple reagents problems, I was not able to finish much of the proposed work. The transfer of the award and the commencement of the new work plan began in October of 2002. I am including a copy of my new statement of work that describes what research I am currently addressing (successfully!).

Statement of Work

The Role of Focal Adhesion Kinase and CAS in Integrin-mediated Signaling on Distinct Forms of Laminin-5

Task 1. Analyze FAK activation and CAS phosphorylation following adhesion of MCF-10A's to pro-migratory, cleaved vs. uncleaved laminin-5 matrix.

a. Phosphorylation/activation state of endogenous FAK will be determined by immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies and in vitro kinase assay following a time-course after adhesion to laminin-5.

Task 1 Progress Report (7/01): Preliminary experiments showed enhanced phosphorylation of both FAK and CAS following adhesion of MCF-10A's to cleaved, pro-migratory laminin-5. These experiments were carried out using immunoprecipitation with specific FAK or CAS antibodies followed by Western blotting with anti-phosphotyrosine antibodies. In vitro kinase assays to measure activation of FAK have not been completed to date.

Task 1 Progress Report (12/01). In vitro kinase assays were not performed.

Task 2. Analyze FAK activation and CAS phosphorylation following stimulation of MCF-10A's with β1-integrin activating antibody TS2/16.

Task 2 Progress Report: In preliminary experiments, I was unable to repeat the previously reported TS2/16-stimulated migration of MCF-10A's on laminin-5. To test if this was due to the batch of antibody that I was using I obtained the TS/216 hybridoma and prepared a fresh stock of the antibody from serum-free cell culture supernatants using a Protein G-Sepharose chromatography. The new stock of antibody has allowed me to repeat the

experiments showing enhanced migration of 10A's on laminin-5. Analysis of FAK and CAS phosphorylation have not been completed.

Task 2 Progress Report (12/01). ***Analysis of FAK and CAS phosphorylation were not performed using the new batch of TS/216.***

Task 3. Determine role of exogenously expressed FAK, CAS and respective mutants on migration of MCF-10A's.

a. Stable cell lines will be generated by transfection of MCF-10A's with expression vectors encoding epitope-tagged versions of FAK, CAS and variants.

Task 3a Progress Report (7/01): Three attempts were made in the past year to obtain stable MCF-10A cell lines over-expressing FAK, CAS and variants. FAK and CAS cDNA's were subcloned into pcDNA3.1 (Invitrogen), a eukaryotic expression vector containing a CMV-promotor and G418 drug resistance marker. In all attempts I was able to obtain numerous G418 resistant clones, but no clones over-expressed either FAK or CAS. The expression vectors were determined to be functional by transient transfection of COS cells. I am currently pursuing transient transfection experiments of MCF-10A cells using these vectors in combination with GFP as a marker for transfection efficiency.

b. Analyze migration of cell lines on laminin-5 and other extracellular matrix molecules.

c. Corroborate data generated from stable cell lines with analysis of same constructs in transient transfection assays.

Task 3a, b, c Progress Report (12/01): Transient transfection studies were not completed.

Task 4. Determine role of migration-linked signaling pathways in laminin-5 cleavage model.

a. Analyze cAMP levels in MCF-10's plated on cleaved vs. uncleaved laminin-5
b. Analyze activation of ERKs during migration on cleaved laminin-5

Task 4 Progress Report (7/01): Task 4 experiments have not been attempted to date.

Task 4 Progress Report (12/01): Studies assessing the activation of ERK upon engagement of MCF10 cells on Laminin-5, Fibronectin, Poly-L-Lysine were performed. Results showed that ERK became phosphorylated upon engagement with all of these substrates, only in the presence of the Beta1 integrin activating antibody TS/216.

Additions to original Statement of Work (7/01):

A) While performing experiments associated with Task 1 it became evident that much variation existed in stocks of cleaved laminin-5 that were produced by enzymatic digestion of purified matrix. To address this issue I am in the process of constructing a recombinant laminin-5 γ2 chain that is truncated at precisely the point of enzymatic cleavage. This construct contains a 6-His tag at the carboxyl terminus and a signal peptide at the amino terminus. A full-length γ2 chain construct with a 6-His tag is also being constructed. The goal is to express either the full-length or truncated molecule in either MCF-10A or the rat bladder carcinoma cell line 804G, which both express the laminin-5 β3 and α3 chains. Laminin-5 will be purified from cell culture supernatants using metal chelate affinity chromatography. Using this system I should be able to obtain laminin-5 preparations that only contain the cleaved or the uncleaved γ2 chain. Potential problems with this system are: 1) Improper or non-functional association of exogenously expressed truncated γ2 chain with endogenous α and β chains. However, evidence from the literature suggests that the exogenously expressed γ2 chain should associate normally with the endogenous α and β laminin chains. 2) Inability to purify sufficient quantities of recombinant laminin-5 using this system.

Progress Report on Addition 1 (12/01):

The His tagging and the fusion of a signal peptide sequence at the amino terminal cleavage site were performed. When I attempted to assemble these pieces into a full-length construct for testing I found multiple errors in the restriction enzyme map that I had been sent. Subsequent sequence analysis revealed that this cDNA was not what it was said to be. Ultimately, I ran out of time to finish this project.

B) Laminin-5 is not commercially available at this time. We have relied on an industry resource to obtain this material for our experiments. So I would not be dependent on this source alone, I have set up a system to purify laminin-5 from 804G cell culture supernatants using an antibody affinity column. (laminin-5 γ2 chain specific monoclonal antibody TR1). I have been able to isolate pure, soluble laminin-5 using this system and will use this as a back up if needed.

Progress Report on Addition 2 (12/01): Laminin-5 was purifed as described. However, I found that these cells not only secrete laminin-5, but they secrete a significant amount of fibronectin as well. This can be removed from the preparations by a preclearing step with a fibronectin antibody affinity column. This is an important factor in interpreting data from any studies done on matrix secreted by these cells and was something I was not aware of when I began this purification.

Key Accomplishments June 30, 2000 – July 31, 2001:

- Analysis of FAK and CAS phosphorylation following adhesion of MCF-10A's to promigratory, cleaved vs. uncleaved laminin-5.
- Determination that MCF-10A cells will not tolerate exogenously expressed FAK, CAS and variant molecules.
- Partial construction of a recombinant cleaved laminin-5 γ2 chain cDNA.
- **Publication of manuscript cited below.**

Key Accomplishments August 1, 2001 – December 15, 2001:

- Began analysis of ERK phosphorylation in MCF10A cells on laminin-5 in the presence of TS/216 antibody.
- Performed fluorescence analysis of MCF10A cells on Laminin-5.

Reportable Outcomes June 30, 2000 – July 31, 2001.

Publications and Abstracts:

Earley, B., Plopper, G.E. and J.L. Huff. The role of FAK in breast cell migration on laminin-5. Western Alliance of Medical Students Annual Meeting 2001.

Plopper, G.E., J.L. Huff, W.L. Rust, M.A. Schwartz and V. Quaranta. 2001. Antibody induced activation of β1 integrin receptors stimulates cAMP-dependent migration of breast cells on laminin-5. *Molecular and Cellular Biology Research Communications* **4**, 129-135.

Reportable Outcomes August 1, 2001 – December 15, 2001:

Publications: These projects were from graduate students that I helped train during the course of this award.

Wagner, J.E. , Huff, J.L., Rust W.L., Kingsley K., and Plopper, G.E. 2002. Perillyl alcohol inhibits breast cell migration without affecting cell adhesion. *J. Biomed. Biotechnol.* **2**, 136-14.

Kingsley, K., W.L. Rust, J.L. Huff, R.C. Smith and G.E. Plopper. 2002. PDGF-BB enhances expression of, and reduces adhesion to, laminin-5 in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **294,** 1017-1022.

Kingsley, K., J.L. Huff, W.L. Rust, K. Carroll, A. Martinez and G.E. Plopper. 2002. ERK1/2 mediates PDGF-BB stimulated vascular smooth muscle cell proliferation and migration on laminin-5. *Biochem. Biophys. Res. Commun.* **293,** 1000-1006.

Patent Application: Method and System for In Vitro Screening of Compounds For Pro/Anti-Migratory Effects. Serial No. 60/183,628

Student Training (7/01):

Brian Earley, undergraduate Biology major, did an independent research project under my supervision from May 2000 to March 2001. He submitted an abstract on his project and presented a talk at the Western Alliance Medical Student Conference held in February in Carmel, CA. In addition, Brian was awarded an undergraduate research grant for $1000.00 for supplies from UNLV for work done on this project. Brian is currently applying for admission to medical school.

Kilpatrick Carroll, undergraduate Chemistry major, did independent research under my supervision during the first year of this grant. He worked extensively on the attempts to make MCF-10A cell lines. He is enrolled in graduate school at Columbia University for Fall 2001.

Michael Cascia, undergraduate Biology major, assisted in this project for a 3 month period (March 2001-May 2001). Michael utilized the laboratory research skills that he acquired to obtain employment as a laboratory technician at UCSF.

Conclusions: The analysis of integrin-mediated signaling that occurs in breast cells on distinct forms of laminin-5 was a project frought with numerous technical difficulties, and in addition, the laboratory where this work was being conducted was abruptly shut down when my advisor left for a new position in August of 2001. This award was transferred to BioForce Nanosciences, Inc. in October of 2002 and a new statement of work was approved for the duration of this award.
Technical issues tackled during this project: 1) Failure of integrin activating antibody to reproduce previously reported stimulation of migration. Freshly purified batch of this antibody was prepared and did show the enhanced migration effect, however time did not allow me to complete the research objectives described for use of this antibody.
2) Inability to obtain stable cell lines overexpressing FAK, CAS and variant proteins. Multiple attempts were conducted at obtaining stable cell lines transfected with constructs expressing these proteins, but only G418 resistant, non-expressing clones were obtained. This is a mystery to me still as the expression vectors worked well in a transient system and have worked in other laboratories for production of stable cell lines. It is possible that these constructs were toxic to the MCF10A cell line that I was using.
3) Laminin-5 cleavage products produced by enzymatic digestion of purified laminin-5 were not consistent and of high enough quality for reproducible experimental data on migration and signaling to be obtained. I embarked on the construction of a recombinant laminin-5 gamma-2 chain that was truncated at precisely the point of enzymatic cleavage. This cloning project progressed smoothly until it was revealed that the actual gamma-2 chain cDNA that I had obtained was incorrect. Ultimately, I ran out of time to finish this project.
I have attached the statement of work for the current research that I am conducting at BioForce Nanosciences for the duration of this award.

Revised Statement of Work for DAMD17-00-1-0362
Principal Investigator: Janice L. Huff, Ph.D.

I. Background:

Molecular medicine in the post-genomic era holds the promise of earlier cancer diagnosis and better chemotherapy choices by detecting and cataloging molecular signatures of pre-malignant disease and disease progression. This information will be useful for development of sensitive cancer screening tests, for monitoring clinical disease progression, for defining appropriate drug targets, and for monitoring individual patient's response to chemotherapeutic drugs [1,2,3].

To attain these goals the need exists for assay systems that can simultaneously measure multiple protein species with sensitivity to discriminate subtle quantitative as well as qualitative changes in these molecules and their binding partners. In addition, to be practical and clinically useful for large scale studies these assays must also meet the commercial requirements of speed, cost efficiency and requirement for minimal starting tissue material.

Practical microarray tests for protein-based screening are beginning to emerge. Current protein microarray assays have shown promise in meeting the needs for the type of assay described above [4,5]. In the most common format these assays involve mechanical deposition of suitable capture molecules, such as specific antibodies, in an array format onto an appropriate substrate. Mechanical deposition methods can be subdivided into two categories: controlled spray of microdroplets (i.e., inkjet printing) and pin tool deposition. Both of these approaches deposit nanoliter amounts of starting material, which correspond to molecular domain sizes ranging from slightly smaller than 100 μm^2 to 300μm^2. These arrays are readily multiplexed so that numerous protein targets can be studied in parallel. The reverse phase protein array is a variation on this assay format that was developed by investigators in the FDA-NCI clinical proteomics program to study changes in important cellular regulatory proteins during pre-malignant conversion within a single individual. In this assay format small quantities of protein lysates from laser dissected tissue biopsy samples (LCM) are directly deposited in an array format on nitrocellulose-covered glass slides. These slides are then interrogated with antibodies directed against proteins of interest [6,7]. This format allows direct comparison of changes in protein profiles of tiny, multiple tissue biopsy samples from a single individual and allows subtle changes in protein profiles that accompany pre-malignant conversion to be studied in detail. Although this system requires less starting tissue biopsy material than the more common antibody-capture format [8], it is still limited by the requirement of relatively large amounts of protein necessary for each successful test. *This is a key factor that negatively impacts the practical application of the current methodology for real world cancer biopsy screening where available sample size is vanishingly small.*

BioForce Laboratory, Inc., http://www.bioforcelab.com) is an industry leader in the development of next-generation biomolecular screening arrays, or NanoArrays, with patented technology permitting the construction of biochips with sub-micron spatial addresses. These nano-scale arrays allow a thousand or more molecular tests to be carried out in the same surface space occupied by a single state-of-the-art microarray spot. (See Figures 1 and 2) An obvious advantage of this design is the dramatic savings in reagent cost that accompanies ultra-miniaturization. *More importantly, it provides a realistic platform for performance of array-based analyses in applications involving extremely small quantities of sample material where uses of current microarray formats are not feasible.*

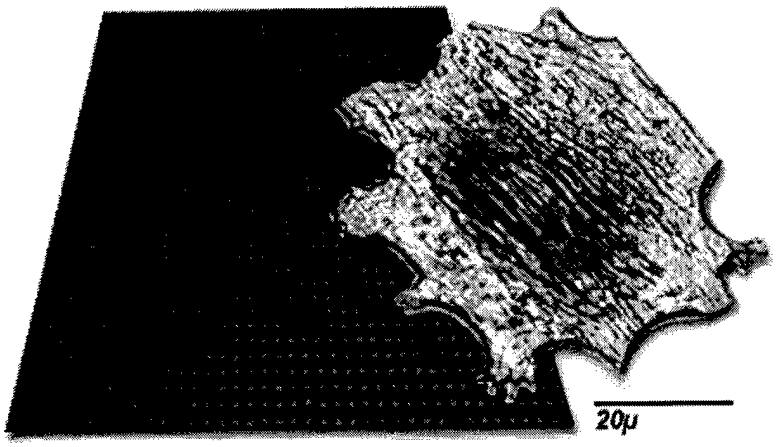
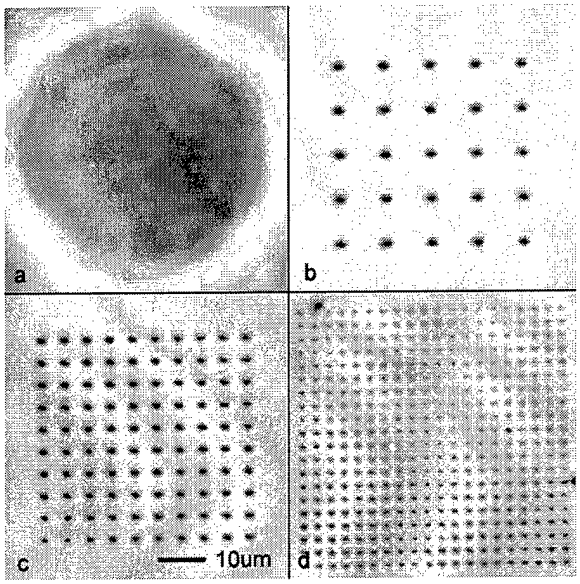


Figure 1: Size comparison of a NanoArray (~1 μm diameter spots at a density of 1 spot/ 4 μ^2, pitch = 3μ) with a typical frog retinal glial cell shown to scale.

Figure 2. Optical micrograph comparison of NanoArray and conventional microarray spot sizes. Panel a shows a 30μm diameter microarray spot generated by MicroJet deposition. Panels b, c and d show NanoArrays with 5 x 5, 10 x 10 and 20 x 20 spot densities, respectively.



The goal of this revised statement of work is to construct a nano-scale protein array platform and use it as a basic research tool to study alterations in cellular signaling pathways that accompany breast cancer disease progression. In the long term we anticipate that this assay will provide a much needed platform for breast cancer proteomics.

II. Specific Aims

Specific Aim 1: Determine precision and linearity of NanoArray platform using purified antigen.

1) A suitable protein, such as recombinant human estrogen receptor (#RDI-ERALPHA-AG Research Diagnostics Inc, NJ) will be arrayed in dilution curves on NanoArray chips and will be detected with an anti-estrogen receptor antibody (Research Diagnostics Inc., NJ), followed by a fluorescently labeled secondary antibody.

   From these control experiments we will determine: Intra- and Inter-spot reproducibility, limits of protein detection, and linearity of detection.

Specific Aim 2: Determine precision and linearity of NanoArray using protein lysates from breast cancer cell lines.

1) Defined cell lysates from standard breast epithelial cell lines representing various stages of clinical breast cancer progression (normal: MCF10A; MCF-7, MDA-MB-231 and MDA-MB-425) will be prepared for use in this phase of assay optimization.
2) Salt, protein and detergent concentrations may be altered to achieve optimal protein deposition.
3) Protein profiles of previously validated markers proteins will be generated during this phase of research. These markers will include Estrogen Receptor, Progesterone Receptor and HER2/*neu*.

Specific Aim 3: Study expression patterns of pro-survival markers in breast cancer progression cell line model.

1) Cell lysates will be prepared, arrayed and processed according to conditions established upon successful completion of Specific Aims 1 and 2.
2) Identically arrayed protein chips will be probed with monoclonal antibodies for pro-survival markers. Specifically, we will assess expression levels and phosphorylation states of Protein Kinase B/Akt and ERK, both protein kinases speculated to be important in regulation of cell proliferation and cell survival.
3) Protein loading will be normalized by measuring actin levels on identically arrayed chips.

III. Research Design and Methods:

A. Deposition substrate:

NanoArrays are constructed on a variety of debris-free and topographically flat substrates; and both chemically reactive and inert surfaces are available for testing.

1) Passive of molecules adsorption onto a gold coated glass or polished silicon wafer is the easiest and most straight forward method of protein deposition. This method works extremely well for arraying purified proteins under low salt conditions, and will be the first method tested for arraying of the cell lysates.

These substrates are prepared as follows: #1 glass cover slips (Fisher Scientific) or polished silicon wafers that were first cut into 4mm X 4mm squares are cleaned thoroughly in water followed by ethanol using an ultrasonic bath for 30 min. Following the cleaning, the substrates are stored in ethanol until used. Surfaces are coated with a thin layer (5 nm) of chromium at 0.1 nm/s followed by the deposition of 10 nm of gold at 0.2nm/ using an IBC 2000 ion-beam sputterer from South Bay Technologies, Inc. Substrates are masked before sputtering with a 200 mesh (lines/in) finder grid containing 105 μm x 90 μm holes. Figure 1 shows a schematic depiction of the layout of a NanoArray chip.

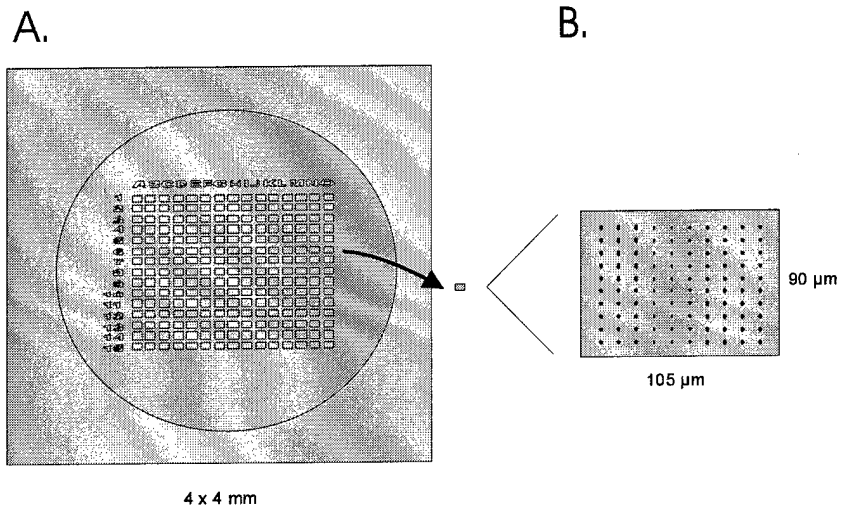


Figure 3. Schematic diagram of NanoArray chip. A. A 15 x 15 finder grid overlayed on a glass coverslip creates a mask which allows easy and accurate sample location on the NanoArray. B. Blow-up of a single 105 μm x 90 μm block showing a 10 x 10 NanoArray.

2) Covalently couple the proteins to a chemically activated surface. Several surface chemistry choices are available:

a. Self assembling monolayer (SAM) of succinimide terminated alkanethiolates on a gold surface. The succinimide groups react spontaneously with primary amines to form a covalent attachment between the surface and the biomolecules in the deposited sample.
b. Aldehyde activated surfaces are generated on mica or flat glass using APTES (aminopropyl triethoxysilane), followed by glutaraldehyde treatment.
c. Versalinx chemical affinity tools (Prolinx, Inc., WA). This is a synthetic, small molecule, low molecular weight affinity pair that is used for surface immobilization of a wide variety of macromolecules. Advantages include ease of use and high retention of ligand activity. Disadvantage, each sample needs to be linked to one member of the affinity pair.

3) Deposition onto hydrophilic polymers and gel-based matrices.

a. Hydrogel chips (Packard, CT) consist of a porous polyacrylamide matrix into which deposited proteins are immobilized. This provides an aqueous environment for subsequent antibody interactions.
b. FastSlides (Schleicher and Schuell, OR) are glass-backed nitrocellulose and are the substrate used in construction of reverse-phase protein microarrays.

Factors that will be considered when chosing an appropriate substrate are: preservation of biological activity of deposited molecules, protein binding capacity, and ease of use.

B. Deposition conditions:

1) NanoArrayer tip composition: We currently use a tip fabricated of silicon nitride for NanoArray deposition. This tip may be modified to increase protein loading and flow.
2) Room humidity: This is the relative humidity in the NanoArrayer housing, and is altered for optimal deposition depending on sample composition.

C. Post-deposition chip processing and optical detection:

1) Hydration: Time, post-deposition, in which the chip hydrates in a humid chamber beforeantibody incubation steps.
2) Standard optimization of antibody incubation steps.
3) Optical detection: Fluorochromes will be chosen to obtain the best signal intensity.

Figure 2 outlines steps in construction of a NanoArray with subsequent optical (fluorescent) readout.

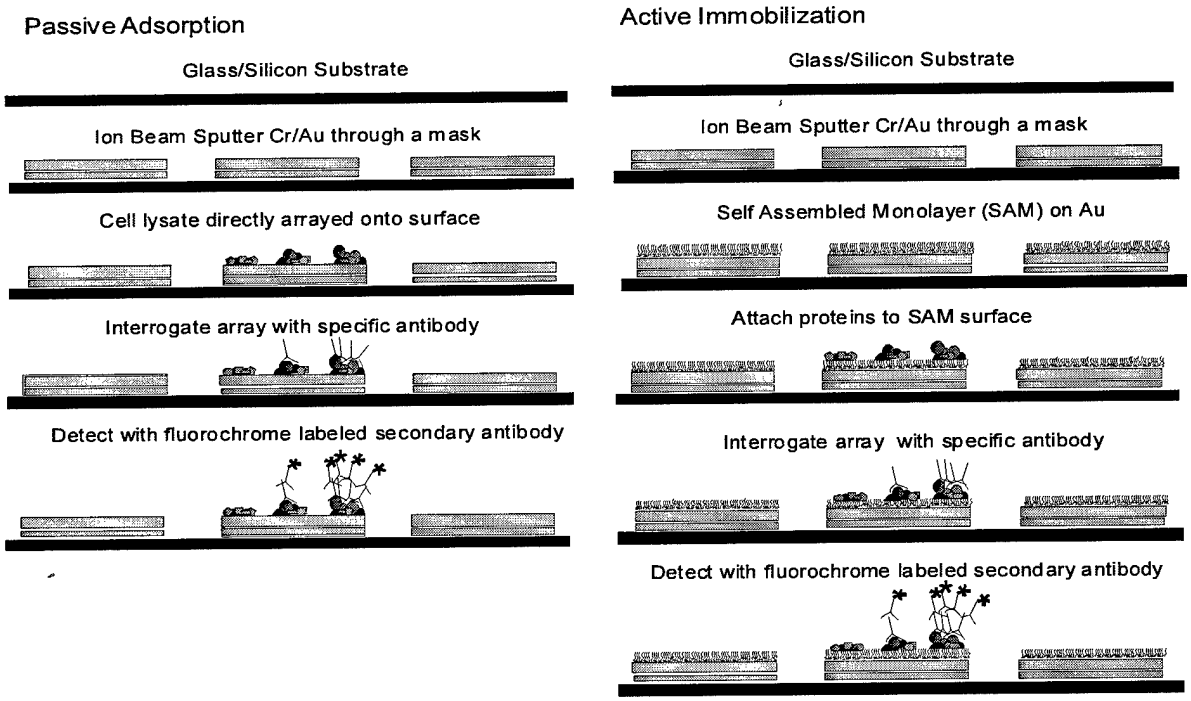


Figure 2. NanoArray construction and optical readout. NanoArrays can be constructed either by Passive adsorption (left side) or by an Active immobilization of the antibodies to the chip surface (right side). In both cases, either glass or silicon chips are first layered with chromium and then gold.

D. NanoArray Data Acquisition and Analysis:

1) Fluorescent image data will be taken from each array and stored in a tagged image file format (TIFF). The P-SCAN software package will be used for image analysis [7].
2) Data will be analyzed for reproducibility by depositing each sample in a minimum of 10 NanoArray spots.

3) The dynamic range of marker detection, or assay sensitivity in terms of cell numbers, will be assessed by analyzing data derived from dilution curves of the cellular lysates.

Project Timeline and Milestones:

<table>
<tr><th></th><th>Specific Aim 1</th><th>Specific Aim 2</th><th>Specific Aim 3</th></tr>
<tr><td rowspan="2">0-6 Months</td><td colspan="2">NanoArray Purified Proteins</td><td rowspan="2"></td></tr>
<tr><td>Deposition Conditions</td><td>Dynamic Range of Marker Detection</td></tr>
<tr><td rowspan="2">6-12Months</td><td rowspan="3"></td><td colspan="2">NanoArray Breast Cancer Cell Lines Known Markers</td><td rowspan="3"></td></tr>
<tr><td>Deposition Conditions</td><td>Dynamic Range of Marker Detection</td></tr>
<tr><td>9-13 Months</td><td colspan="2"></td><td>NanoArray Breast Cancer Cell Lines Pro-Survival Markers</td></tr>
<tr><td>PROJECT MILESTONES</td><td>NanoArrays show reproducible and linear detection of recombinant Estrogen Receptor.</td><td>NanoArrays show reproducible and linear detection of Estrogen Receptor from human breast cell lines.</td><td>NanoArrays are useful for experimental data acquisition.</td></tr>
</table>

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Human Subjects Research: Claim of Exemption Form is attached.
Animal Use: This research does not involve the use of vertebrate animals.

Functional Protein Nanoarrays for Biomarker Profiling

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Keywords: nanoarray, microarray, protein array, cytokine, AFM

Abbreviations: AFM, atomic force microscopy; PCR, polymerase chain reaction; LCM, laser capture microdissection; SAM, self-assembling monolayer; PDMS, poly(dimethylsiloxane); PDBA, 1,3-phenyldiboronic acid; SHA, salicylhydroxamic acid, DSU, dithiobis-succinimidyl undecanoate

Graphical abstract: Figure 1

Abstract

The use of microarrays for parallel screening of nucleic acid profiles has become an industry standard. Similar efforts for screening protein-protein interactions are gaining momentum, however they remain limited by the requirement for relatively large sample volumes. One strategy for overcoming this problem is to significantly decrease the size and consequently the sample volume of the protein interaction assay. We report here on our progress over the last two years in the construction of ultraminiaturized, functional protein capture assays. Each 1 micron spot in these array-based assays covers less than 1/1,000th of the surface area of a conventional microarray spot while still maintaining enough antibodies to provide a useful dynamic range. These "nanoarray" assays can be read by conventional optical fluorescence microscopy as well as by novel label-free methods such as atomic force microscopy (AFM). The size reduction realized by functional protein nanoarrays also creates opportunities for novel applications including highly multiplexed single cell analysis and integration with microfluidics and other "lab-on-a-chip" technologies.

Introduction

The advantages of miniaturization and multiplexing have been realized for nucleic acid analysis [1] and are beginning to bear fruit for protein profiling [2]. The lack of a protein amplification method analogous to PCR exacerbates the unfortunate fact that many important protein biomarkers such as HER-2, PSA and CRP are present at concentrations of 10-100 pg/ml. Additional improvements in assay sensitivity could lead to the discovery of other useful diagnostic biomarkers that currently escape detection. With the emergence of technologies such as Laser Capture Microdissection (LCM), it is now possible to analyze the expressed proteins from a carefully selected subpopulation of cells [3]. Therefore, significant benefits are to be gained by further miniaturization of protein assays that require less capture material, less analyte, and may offer greater sensitivity [4,5]. To create ultraminiaturized nanoarrays [6], instrumentation and methodology must be developed for placing tiny volumes of biomolecules on surfaces with high spatial precision. Several methods exist that are appropriate for this purpose. These include poly(dimethylsiloxane) microcontact printing [7], ink-jet printing [8,9], electrospray deposition [10], mechanical pin-tool deposition [11] and photochemical *in situ* synthesis [12]. However, the use of microcantilever devices has proven to be one of the most productive methods for the deposition of small molecules. Utilizing an atomic force microscope (AFM) probe for this purpose was first demonstrated by Jaschke and Butt in a report demonstrating the creation of patterns of alkanethiols on mica [13]. This process has been further developed for deposition of chemically modified oligonucleotides and proteins [14, 15], as well as for the indirect capture of larger biomolecules including proteins [16].

The direct deposition of macromolecules in sub-micron domains presents novel problems that are not readily overcome by the approaches reported thus far. Dip pen nanolithography (DPN) of large proteins such as antibodies can be a slow process [15] with impractical tip-surface contact times required for transfer. Our approach improves the deposition rate by applying precisely timed bursts of wet or dry air directly to the tip-surface interface. The AFM piezo tube used in DPN experiments has an XY travel of approximately 0.1mm, which limits array size, and

multiplexing options. Mechanical XY stages typically found on scanning probe microscopes have inadequate resolution and repeatability to be useful for this application. The instrument we have constructed replaces the piezo tube with a piezoelectric "inchworm" stage capable of 20 nm resolution over 25 mm of XY travel. While not ideal for high resolution DPN, this type of stage excels at performing repeatable movements of several centimeters for the purposes of washing and reloading the probe. We have studied the feasibility of direct deposition of large molecules to create ordered nanoarrays of functional proteins and report here on the methodology and novel instrumentation developed in the course of these studies.

Materials and Methods

Instrumentation. The results reported here were all obtained using the second generation of an instrument we have constructed and termed the NanoArrayer [6]. The basic principle of the NanoArrayer is mechanically mediated direct deposition of materials on surfaces with high spatial precision, surface sensing capabilities and environmental control. Initial development of the first hand crafted NanoArrayer (NanoArrayer I) was promising and led to development of a second generation NanoArrayer (NanoArrayer II), which incorporated closed loop motion control, environmental controls, and surface sensing. A third version (NanoArrayer III) has since been developed and the full details of the NanoArrayer instrumentation development will be reported elsewhere [17]. Briefly, a microfabricated deposition tool is loaded by immersing the distal end in a drop of the protein solution. The surface to be patterned is positioned on a piezoelectric inchworm-driven XY stage with 20 nm resolution over 25 mm of travel (Burleigh EXFO). Manipulating the local humidity at the interface between the deposition tool and surface with gentle bursts of wet or dry air allows precise control of the molecular transfer rate. All stage movement and patterning parameters are controlled from within a custom software package called NanoWare™.

In these proof-of-principle experiments, the deposition tools used to physically place proteins on the substrates were 0.58 N/m DNP AFM probes from Digital Instruments. AFM

probes were pretreated with UV and ozone for 30 min in a UV-TipCleaner (BioForce Nanosciences) to remove silicone oils and organic debris. While adequate for these demonstrations, commercially available AFM probes have proven to be less than ideal for macromolecular deposition. Therefore, non-AFM probe deposition tools for the NanoArrayer are currently under development [18].

Surfaces. The nanoarrays presented here were created on 4 mm squares of diced silicon wafer (Montco Silicon Technologies, Inc.) that were first coated with 5 nm of chromium and 10 nm of gold by ion beam sputtering through an alphanumeric indexed electron microscopy specimen grid (Electron Microscopy Sciences). Gold-coated silicon wafers or "chips" were removed from the sputterer and used for protein deposition (Figure 1) or immediately immersed in alkanethiolate solutions to allow self-assembling monolayer (SAM) formation as described below.

Protein A/G chips were fabricated by overnight incubation of the sputtered gold chips in 0.5 mM solutions of dithiobis-succinimidyl undecanoate (DSU) [19] in 1,4-dioxane. Following successive washes in dioxane and dry 100% ethanol, the chips were blown dry with argon and used for deposition (Figure 4) or allowed to react with 0.1 mg/ml Protein A/G (Pierce) in PBS for 30 min at 25°C. Unreacted succinimide groups were blocked with 10 mM Tris pH 7.5, 1 M glycine for 30 min at 25°C. Without washing, chips were transferred into StabilGuard (Surmodics) protein stabilizing and blocking agent and incubated for one hour at 25°C. Protein A/G chips were then rinsed briefly with a few drops of ddH_2O, dried with argon, and stored in a desiccator at 4°C prior to use (Figure 2, 3).

To make the Versalinx™ SHA SAM chips shown in Figure 5, fresh gold-coated silicon wafers were incubated in Versalinx™ (Prolinx) salicylhydroxamic acid (SHA) alkanethiolate SAM reagents and ethanol as directed by the manufacturer's protocol, then sonicated and washed successively in ethanol and double distilled water (ddH_2O) prior to use. The Versalinx™ SHA SAM chips have been specifically engineered by their manufacturer to bind molecules

functionalized with 1,3-phenyldiboronic acid (PDBA) while exhibiting low non-specific binding of molecules without a PDBA functional group.

Proteins. The antibodies deposited in the nanoarrays in Figures 2-4 were a monoclonal mouse anti-human interferon-γ at 0.9 mg/ml in 10 mM Tris-HCl pH 8.0, 10 mM NaCl (Pierce Endogen) and a monoclonal mouse anti-human IL-6 at 0.9 mg/ml in 10 mM Tris-HCl pH 8.0, 10 mM NaCl (Pierce Endogen). The cytokine sandwich assays in Figure 3 and Figure 4 were performed using matched mouse monoclonal antibody pairs and recombinant proteins generously provided by Pierce Endogen. Our ELISA data from these pairs of antibodies (not shown) indicates high specificity and minimal cross-reactivity with other cytokine matched pairs. The goat IgG used in the AFM height assay (Figure 4) was obtained from BioDesign at 3 mg/ml in PBS, and the rabbit anti-goat was obtained from Sigma at 2 mg/ml in PBS.

Antigen-Antibody Nanoarray. Following the arrayed deposition of IgG onto a Protein A/G chip, the chip was stored overnight in a humid environment at 25°C to facilitate binding of antibody Fc regions to the Protein A/G. The remaining Fc binding sites were blocked by incubation with 5 µl of 5 mg/ml bovine IgG (Sigma) for 30 min at 25°C, followed by a 5 min wash in PBS + 0.1% Tween-80 (PBST). Bovine IgG was found to block the Protein A/G surfaces more efficiently than either rabbit IgG, or rabbit IgG Fc fragments (data not shown). The chip was then immersed in 500 µl of PBST + 1.5 µg/ml Cy3-goat anti-mouse (Jackson ImmunoResearch Laboratories) for 30 min at 37°C. Three successive washes were carried out for 3 min each in 500 µl of PBST prior to image acquisition.

Cytokine Sandwich Assay Nanoarray. Overnight hydration of the mouse antibody arrayed chip at 25°C and 60% RH facilitated complete binding of the mouse antibody Fc domains to the Protein A/G surface. The remaining Fc binding sites were blocked by incubation with 5 µl of 5 mg/ml of bovine IgG for 30 min at 25°C, followed by a 5 min wash in PBST. Bovine IgG was found to block the Protein A/G surfaces more efficiently than either rabbit IgG, or rabbit IgG Fc

fragments (data not shown). Chips were incubated with 5 ng/ml recombinant human interferon-γ (Pierce Endogen) in PBS + 4% IgG-free, protease-free BSA (Jackson ImmunoResearch Laboratories) with agitation for one hour at 25°C. Biotinylated mouse anti-IFN-γ detection antibody (Pierce Endogen) was added to the reaction to 250 ng/ml and incubated with agitation for another hour at 25°C. The chip was then transferred into 500 µl of Cy3-streptavidin (Jackson ImmunoResearch Laboratories) at 1.8 µg/ml in PBST and incubated with agitation for 30 min at 25°C. Three successive washes were carried out for 1 min each in 500 µl of 50 mM Tris pH 8.0 + 0.2% Tween-20 prior to image acquisition.

Cytokine Cross Reactivity Nanoarray.

The multiplexed IFN-γ and IL-6 nanoarray was constructed on a reactive DSU chip and processed using appropriate protocols. Overnight hydration of the mouse antibody arrayed chips at 25°C and 60% RH facilitated complete binding of the mouse antibodies to the succinimide surface. The remaining succinimide groups were blocked by incubation with 5 µl of 100 mM methoxy-PEG-NH_2 (Nektar Therapeutics) in water for 5 min at 25°C, followed by a 30 min wash in PBST. The chip was incubated with 1 ng/ml recombinant human interferon-γ (Pierce Endogen) in PBST with agitation for one hour at 25°C. Biotinylated mouse anti-IFN-γ and anti-IL-6 detection antibodies (Pierce Endogen) were added to the reaction to 250 ng/ml and incubated with agitation for another hour at 25°C. The chip was then transferred into 500 µl of Alexa Fluor 594-streptavidin (Molecular Probes) at 2 µg/ml in PBST and incubated with agitation for 30 min at 25°C. Three successive washes were carried out for 5 min each in 500 µl of PBST prior to image acquisition.

AFM Height Assay Nanoarray. Goat IgG was modified with 1,3-phenyldiboronic acid (PDBA) using the Versalinx™ Amine Modifying Reagent (NHS-PDBA) according to the manufacturer's (Prolinx) protocol, and then dialyzed into 2.5 mM NaCl and recovered at 0.1 mg/ml. After the array deposition was complete, the chip was stored in a humid environment for one hour to allow

for binding of the PDBA-labeled IgG to the Prolinx SHA SAM surface. The chip was washed with 500 µl of PBS + 1% Tween-80 for five minutes, and ddH_2O for five minutes to remove salts and detergent prior to drying and AFM imaging. Incubation with rabbit anti-goat was performed overnight at +4°C in a 1 µl volume at 200 µg/ml in PBS + 1% Tween-80. The chip was then washed five minutes in PBS + 1% Tween-80, five minutes in ddH_2O, and dried prior to final AFM imaging.

Detection. Nanoarrays that utilized fluorescent reporting molecules were visualized on a Nikon TE-2000U inverted microscope equipped with a HYQ Cy3 filter #41007a and a Texas Red filter #41004 from Chroma Technology. Images were captured using a Cohu Model 4922 cooled CCD camera. Immediately prior to visualization, chips were briefly rinsed with a few drops of ddH_2O and inverted onto a drop of n-propyl gallate/glycerol anti-fade solution (5% n-propyl gallate, 70% glycerol, 25% 0.5M Tris-HCl pH 9.0, then adjusted to a final pH of 7.4). Fluorescent images were analyzed for spot size, intensity, and coefficient of variance with the Array Pro Analyzer software package (Media Cybernetics). AFM images were recorded in tapping mode with a Dimension 3100 AFM from Digital Instruments.

Results

The direct deposition of functional proteins in micron and sub-micron domains represents a process that is amenable to parallelization and high throughput with a minimum of steps. Figure 1 shows a brightfield image of a single microarray spot (~55 µm diameter) and three protein nanoarrays of various spot densities. In this demonstration a single protein species was printed multiple times in 1 µm diameter domains at a rate of about one spot per second. Further advances in the development of the NanoArrayer instrumentation and arraying protocols have reduced the tip-surface contact time required for protein transfer to less than 100 milliseconds. The short contact time required to print these spots contrasts with dip-pen nanolithography which is reported to require contact times of up to 250 seconds per micron to achieve protein transfer [15]. The typical reduction in surface area per nanoarray spot relative to microarray spots is

several thousand fold, with estimated sample volumes of approximately 30 attoliters per spot. These spatial and volume dimensions suggest that protein nanoarrays are compatible with picoliter volumes of analyte, which is consistent with the volume of a single cell.

The nanoarray in Figure 2 illustrates that proteins deposited in nanoarrays retain their antigenic properties and will capture antibodies directed against those epitopes. In this experiment, antigen (mouse IgG) was spotted in a 5 x 5 nanoarray with ~700 nm spot diameters (10.5% CV) and a 5 μm pitch (spot to spot spacing) on a Protein A/G surface. The total dimensions of this nanoarray are 20 μm x 20 μm. This mouse IgG nanoarray was incubated with a Cy3-labeled goat anti-mouse antibody and visualized with a fluorescence microscope. The mouse IgG was clearly labeled by the fluorescent anti-mouse antibody, with minimal non-specific background binding. The coefficient of variance (CV) for the net intensity, excluding the outlier in the lower right corner, was 12%. This CV is comparable to typical values reported for protein microarrays.

To test whether proteins deposited in nanoarrays retain their biological activity we constructed a 10 x 10 nanoarray containing an oriented antibody directed against the human cytokine interferon-γ. The total dimensions of this nanoarray are 45 μm x 45 μm. Proper orientation of the capture antibodies was achieved by spotting them on a monolayer of Protein A/G, which binds specifically to the Fc region of IgG and leaves both Fab regions available for antigen binding. The nanoarray was incubated with recombinant human interferon-γ at a biologically relevant concentration (5 ng/ml), followed by labeling with a biotinylated detection antibody that recognized an epitope different than that of the capture antibody. Addition of Cy3-streptavidin allowed detection of the molecular complexes. The presence of fluorescence in the captured image (Figure 3) demonstrated that the interferon-γ sandwich assay was functional and indicated that the capture antibodies in the nanoarray retained their biological activity. Quantitation revealed a 12.4% CV in net spot intensity and a 12.8% CV in area.

Specificity was demonstrated by repeating the cytokine assay with a multiplexed nanoarray containing capture antibodies against both IFN-γ and IL-6. The antibodies in this experiment are not optimally oriented and exhibit a lower specific net intensity because they were arrayed onto a DSU surface rather than the Protein A/G surfaces shown in Figures 2 and 3. Antibody spots with 400 nm diameters and 2 μm spacing were constructed as previously described. The high precision XY stage of the NanoArrayer™ was critical for proper alignment of the spots after washing the probe and loading the second antibody. Incubation of the nanoarray in 1 ng/ml recombinant IFN-γ and subsequent analysis of the fluorescence data from the entire nanoarray revealed a specific net intensity of 14.1 for the IFN-γ spots compared to 3.6 for the IL-6 spots. The net intensities and standard deviations are shown in Figure 4, with a 2 x 2 subsection of the interdigitated nanoarray displayed in the inset.

Nanoarrays occupy a spatial domain that easily traverses traditional optical methods as well as emerging analytical methods such as AFM. AFM offers unique advantages as a label-free, real-time method for analyzing molecular interactions on nanoarrays in physiological solution, thus negating the necessity of amplifying the components of the assay (e.g., culture, PCR, protein expression). The AFM is capable of single molecule sensitivity and 5 orders of magnitude of spatial resolution from 1 nm to 100 μm. Figure 5 shows a nanoarray assay analyzed by AFM.

In this experiment relatively large spots (7 μm) of PDBA-modified goat IgG were deposited in an array on a Versalinx™ SHA-SAM surface (Prolinx). The dried array was scanned in the AFM using tapping mode [19]. One line of spots is shown in Figure 5. The left-hand panel of the figure shows the spot morphology and topography before addition of a second antibody. The average antibody spot height was approximately 6 nm. Rabbit anti-goat antibody was added to the array and allowed to incubate overnight. The array was washed and dried, and again imaged by AFM. The right-hand panel in Figure 5 shows that the spot heights had increased to approximately 12 nm as a result of formation of the antibody-antigen complex on the array. Since

the spots are measured relative to the surrounding area, the 6 nm height increase indicates that there was negligible non-specific background binding to the Versalinx™ surface.

Discussion

Nanoarrays require extremely small amounts of materials for testing. This is a key advantage that promotes further miniaturization of array-based bioanalytical tests from typical microarray dimensions. The surface area of a single nanoarray spot such as those shown in Figure 1 is about 4,500 times less than that of a conventional microarray spot. Picoliter volumes of analyte, which correspond to the smallest volumes deliverable by microjet methods, are easily sufficient to cover a 10 x 10 nanoarray and carry out 100 individual tests. In fact, given the small volume sample handling capability, nanoarray assays could be carried out on the volume equivalent of a single cell. This substantial reduction in sample volumes is mirrored in the reduction in quantities of materials necessary for construction of nanoarrays. The amount of protein in one microarray spot (100 μm in diameter) is adequate for construction of many thousands of nanoarray spots. The importance of this is especially relevant when materials to be deposited are precious, as with many combinatorial drug candidate libraries and unique biological samples. One example of a unique sample could be protein extracts generated from just a few cells of interest harvested by laser capture microdissection (LCM) from a cancer biopsy for construction of reverse phase protein arrays [21].

Protein A/G surfaces, while useful for properly orienting antibodies and boosting specific net intensity, are not trivial to block when used in a sandwich assay as the detection antibodies contribute to an increased background signal. Through the course of our work, we have discovered that incubation of the surface with a high concentration of bovine IgG is sufficient to block the remaining Protein A/G binding sites. This approach produced lower backgrounds than blocking with either mouse IgG or mouse Fc fragments (data not shown). We are pursuing further improvements to this blocking protocol, which will form the basis for a short techniques manuscript in the near future.

How small is small enough? By our rough estimates, the useful limit for an individual antibody capture domain is 250 nm. Single molecule detection systems are seductive and offer novel insight into the properties of molecules. However, they present difficult statistical issues when it comes to real world assays. This is a result of the dynamic nature of individual molecules and the requirement for many sampling events or lengthy sampling times of a single molecule to obtain a robust diagnostic readout. Hence, there is a dimensional "sweet spot" in terms of domain size for nanoarrays. Below a certain size threshold the spots will lack an adequate number of active and properly oriented capture molecules, and may suffer from inaccurate quantitation and poor dynamic range. The numbers of proteins present within various spot sizes are estimated in Table 1. For a typical IgG, the number of molecules packed into a 1 micron diameter spot is on the order of 10^4, and the number of molecules in a 250 nm diameter spot is on the order of 10^3. If a detection system with single molecule sensitivity such as the AFM is employed, nanoarrays can potentially provide a dynamic range of three to four orders of magnitude. This is sufficient to permit robust concentration analysis and diagnostic confidence. Capture domains below the 250 nm size threshold may lack a sufficient number of molecules to offer reliable quantitative data and a useful dynamic range.

To fully capitalize on the benefits of the ultra-miniaturized nanoarray format, the analyte must be constrained to the smallest possible volume relative to the area occupied by the capture domains. According to Einstein's 'square root law' of Brownian motion [22], $< |r|^2 > = 6\ D\ t$, the distance of molecular diffusion, r, will be proportional to the square root of the time, t, and therefore a modest reduction in volume will yield a significant decrease in diffusion time. This reduction in array size ensures that statistically every analyte molecule will have an opportunity to sample the entire capture surface in a reasonable amount of time. A traditional microarray utilizing tens or hundreds of microliters of sample spread out across several centimeters of capture domains results in the effective wasting of the majority of the sample because it will never be in close proximity to a relevant capture domain unless additional energy is added to enhance

the movement of the analyte (e.g., agitation, mixing or electromotive force). In a typical static two hour incubation, each microarray spot theoretically samples no more than a 1.7 mm radius half-sphere. In contrast, a multiplexed nanoarray in a tiny microfluidic chamber measuring 100 μm x 100 μm could expose each capture domain to every analyte molecule in a matter of minutes. Extended incubation times would increase sensitivity by taking further advantage of a mass-sensitive rather than concentration-sensitive phenomenon referred to as "analyte harvesting" [4]. Current techniques are inadequate for efficient handling of the nanoliter sample volumes proposed here, however sophisticated microfluidic systems capable of addressing this need are in various stages of development and commercialization [23].

A desirable feature of protein nanoarrays is their ability to be analyzed by conventional optical methods. The small size of nanoarrays allows them to be more accessible optically than microarrays since the entire nanoarray field can be read in a single optical microscope image, eliminating the need for specialized optical readout instrumentation. Moreover, in a typical 100 μm diameter microarray spot scanned in a microarray scanner, pixel density is on the order of ~300 pixels/spot at 5 μm resolution or ~80 pixels/spot at 10 μm resolution. In comparison, using an optical microscope with a 1.3 megapixel cooled CCD camera, the pixel density for a 1 μm nanoarray spot is about ~50 pixels/spot when viewed with a 60x objective or ~110 pixels when viewed with a 90x objective, sufficient for statistically significant sampling of each nanoarray spot by optical microscope.

In addition, as demonstrated in Figure 5, nanoarrays are sufficiently small to allow analysis by novel methods that may offer unique benefits of their own. AFM, for example, is a label-free direct readout methodology that has the potential for single molecule sensitivity and simultaneous acquisition of multiple data sets (topography, binding force, viscoelasticity, etc.).

This report describes the first steps in real-world utilization of protein nanoarrays. Perhaps the most exciting aspect of nanoarrays is that their small size creates opportunities for

novel applications that are limited not by spatial scale but rather by the researcher's imagination and ingenuity.

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Figure 1. Brightfield optical comparison of microarrays vs. nanoarrays. The upper left panel shows a state-of-the-art protein microarray spot created with a piezo-jet device (~55 μm diameter). The remaining panels show protein nanoarrays at various spot densities. Nanoarray spot sizes are 1-2 μm in diameter with the pitch (spot to spot spacing) kept greater than 2 μm to ensure that the individual spots can be resolved optically. Higher spot densities and smaller pitch and diameter values are possible, however at spots sizes below about 250 nm the number of molecules per spot is so small that the statistical robustness of the assay becomes suspect.

Figure 2. Antigen – antibody nanoarray. A 5 x 5 nanoarray of mouse IgG as a target protein was deposited in 700 nm diameter spots with a 5μm pitch. The spots were then interrogated with a Cy-3 labeled goat anti-mouse IgG. The presence of fluorescence at the mouse IgG domains indicates that the mouse epitopes were still antigenic.

Figure 3. Functional cytokine sandwich assay nanoarray. Anti-interferon-γ capture antibody was spotted in 1.0 μm diameter domains with a 5 μm pitch on a Protein A/G surface. The nanoarray was interrogated with 5 ng/ml interferon-γ in PBS + 4% BSA, then incubated with a second, biotin-labeled anti-interferon-γ antibody. Cy3-streptavidin was used to facilitate detection of the sandwich complexes. Fluorescence indicates successful binding of interferon-γ by active, oriented antibodies at each anti-interferon-γ domain, followed by interferon-γ epitope recognition by the second antibody.

Figure 4. Specificity of cytokine antibodies in a multiplexed nanoarray format. The inset is a 2 x 2 subsection of a larger array containing capture antibodies directed against IFN-γ and IL-6 that was exposed to 1 ng/ml IFN-γ. Analysis of the fluorescence data from the entire nanoarray revealed the net spot intensities and standard deviations as shown. The spots have a mean diameter of approximately 400 nm and a 2 μm pitch.

Figure 5. AFM assay of a multi-layer protein-protein interaction assay in the nanoarray format. Average height of each spot of goat IgG increased from 6 μm to 12 μm after binding of the rabbit anti-goat antibody.

Table 1. Estimated maximum number of proteins per spot, for a given spot diameter and protein parking area, assuming an ideally packed monolayer. Our assumptions are based on reported IgG dimensions of 14.5 nm long x 8.5 nm wide x 4 nm thick, a minimal parking area of 34 nm^2 and a maximal parking area of 123 nm^2.

Figure 1.

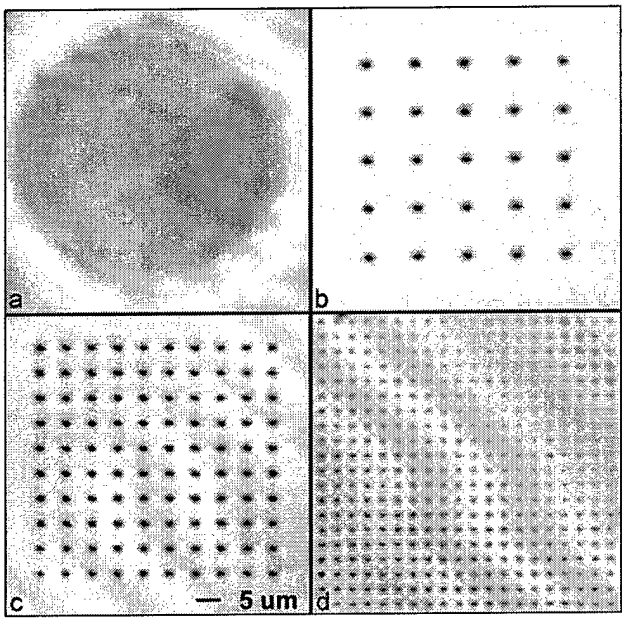

Figure 2.

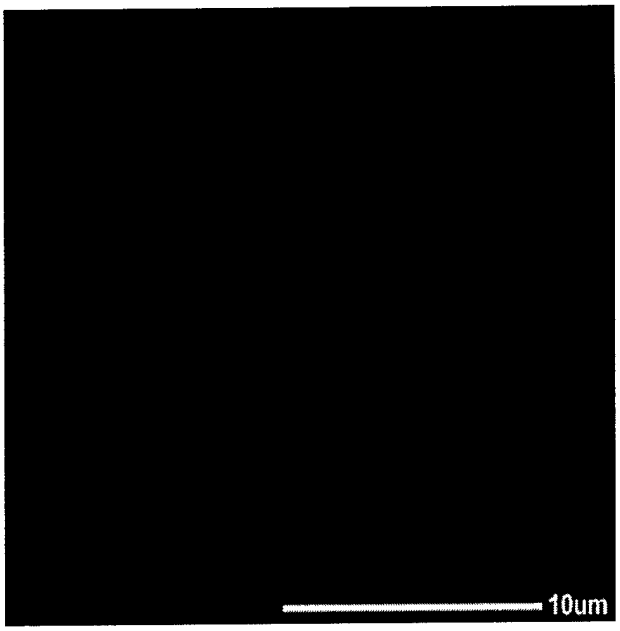

Figure 3.

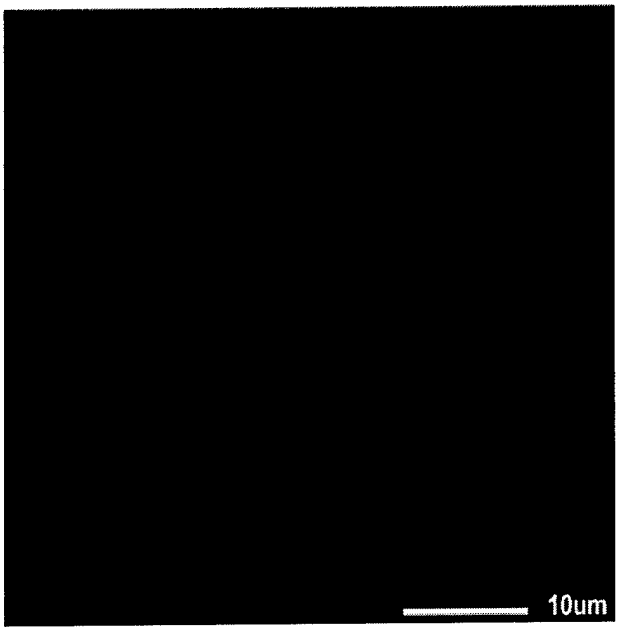

Figure 4.

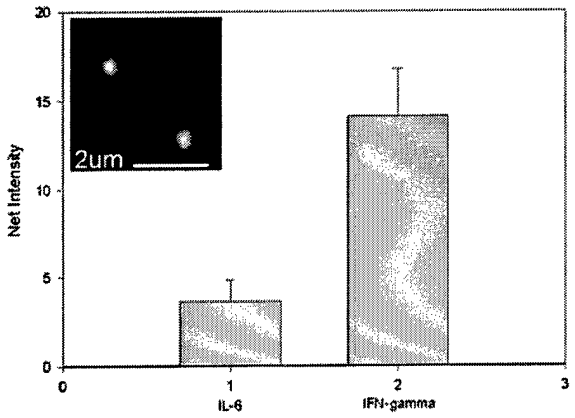

Figure 5.

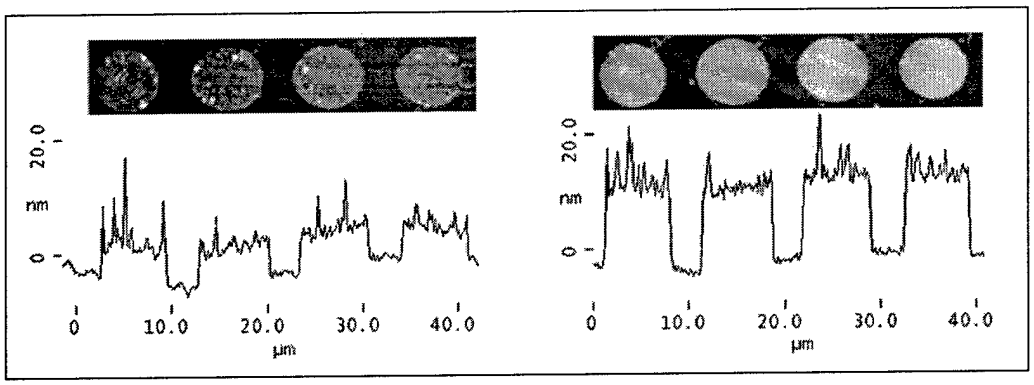

Table 1.

Spot Diameter (nm)	Protein Area (nm^2)				
	36	**45**	**100**	**125**	**150**
50	55	44	20	16	13
100	220	170	80	60	50
250	1,400	1,100	490	390	330
500	5,500	4,400	2,000	1,600	1,300
1,000	22,000	17,000	7,900	6,300	5,200
5,000	550,000	440,000	200,000	160,000	130,000
10,000	2,200,000	1,700,000	790,000	630,000	520,000
50,000	55,000,000	44,000,000	20,000,000	16,000,000	13,000,000
100,000	220,000,000	170,000,000	79,000,000	63,000,000	52,000,000